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Entry

Cyclodextrin-Based Nanosponges and Proteins

Silvia Lucia Appleton [1,†], Yousef Khazaei Monfared [1,†], Francisco José Vidal-Sánchez [2], Fabrizio Caldera [1], Roberta Cavalli [3], Francesco Trotta [1,*] and Adrián Matencio [1,*]

[1] Dipartimento di Chimica, Università di Torino, 10125 Torino, Italy; silvialucia.appleton@unito.it (S.L.A.); yousef.khazaeimonfared@unito.it (Y.K.M.); fabrizio.caldera@unito.it (F.C.)
[2] Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Regional Campus of International Excellence "Campus Mare Nostrum", Universidad de Murcia, 30100 Murcia, Spain; fjose.vidal@um.es
[3] Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, 10125 Turin, Italy; roberta.cavalli@unito.it
* Correspondence: francesco.trotta@unito.it (F.T.); adrian.matencioduran@unito.it or adrian.matencio@um.es (A.M.)
† These authors contributed equally to this work.

Definition: Cyclodextrin-based nanosponges (CD-NSs) have gained importance in drug delivery in the last years due to their easy synthesis and versatility. However, their use as carriers for the delivery of macromolecules such as proteins is less known and sometimes difficult to consider. In this entry, the authors summarize and highlight the multiple possibilities of CD-NSs to deliver active proteins, improving their activity or stability. Starting with a brief description of CD-NSs and their characteristics, the entry will be focused on several proteins, such as (1) Lipase, (2) Insulin and (3) Nisin, for chemical or pharmaceutical applications. The revised results demonstrated that CD-NSs can generate different and interesting applications with proteins. These results could be added to their uses with small drugs, being an interesting alternative for protein delivery and applicability.

Keywords: cyclodextrin based nanosponges; protein; enzyme; Lipase; Peroxidase; Insulin; Nisin

Citation: Lucia Appleton, S.; Khazaei Monfared, Y.; Vidal-Sánchez, F.J.; Caldera, F.; Cavalli, R.; Trotta, F.; Matencio, A. Cyclodextrin-Based Nanosponges and Proteins. *Encyclopedia* **2022**, *2*, 752–760. https://doi.org/10.3390/encyclopedia2020052

Academic Editors: Jose L. Arias and Raffaele Barretta

Received: 18 February 2022
Accepted: 6 April 2022
Published: 13 April 2022

1. Introduction

Although novel drugs still arrive on the market every day, they usually present problems concerning their solubility and stability. For years, the use of cyclodextrins (CDs) to carry small drugs has been exploited. CDs are well-known to the scientific community for their uses in solubilizing poor-soluble drugs [1–3]. Chemically, CDs are cone-shaped oligosaccharides obtained from starch with α-(1,4) linked glucose units. The most common CDs are the natural derivatives with six, seven and eight glucose units called α, β and γ-CD, respectively. The CD ring is a conical cylinder of an amphiphilic nature, with a hydrophilic outer layer (formed by the hydroxyl groups) and a lipophilic cavity [4]. Classically, poorly-soluble drugs are complexed with CD, creating the so-called "inclusion complex", which increases its solubility, stability or bioactivity [5–7].

In some cases, their capacity lacks in efficiency due to the complex structure of the drug or the desire of different profiles (e.g., slow release), and different materials have been prepared to improve their properties, such as the cited Cyclodextrin-based Nanosponge (CD-NS), an innovative cross-linked polymer with a three-dimensional network and a tunable structure with crystalline, amorphous or spherical structure and possessing good swelling properties [8]. Recent reviews [9–12] indicate their wide potential and negligible toxicity [13,14], increasing their biocapacities in several applications, including the ability to (i) improve the apparent solubility of poorly soluble drugs, (ii) modulate drug release and activity, (iii) protect drugs against several agents, (iv) enhance bioactivities, (v) absorb contaminants or deliver the drug, etc.

In recent years, the use of proteins in industry or in therapy has increased, creating an interesting atmosphere for CD-NS, to study all of the positive effects they have on both small molecules and larger ones. A specific case could be the use of CDs with proteins to prevent their aggregation or crystallization of amorphous stabilizers during freezing, even acting as artificial chaperons [14,15]. However, the entire protein cannot be captured by CDs, only by different motifs, which creates different protection zones. In this context, the supramolecular form of CD-NS could generate some benefits by capturing the entire protein within its 3D network.

This entry will try to summarize, explain and emphasize, simply but scientifically, the different opportunities and novelties of CD-NS in protein stabilization using different examples; Dioxygenase [16], Bovine Serum Albumin [17], Lipase [18], Peroxidase [19], Lysozyme [20], Insulin [21] and Nisin [22,23] after explaining the types and characteristics of different CD-NSs for eventual use of the reader.

2. Cyclodextrin-Based Nanosponges' Synthesis and Classification

This section provides a brief description of nanosponges and their classification; for further information, there are excellent chapters and reviews available elsewhere [11,24].

2.1. General Synthesis Protocol

There are some synthetic conditions required to produce both non-water-soluble and water-soluble CD-NSs (Figure 1) [9,25,26]. In this last case, working in conditions that disrupt the limits of native CDs, and particularly aqueous solubility in the combination of a specific solvent, led to the production of a three-dimensional reticulated network associated with the interstitial spaces among monomers [27]. The ultimate structure of this network and its density is highly dependent on the number and type of crosslinker, as well as the ratio with CDs [28]. In addition, the pockets of the polymer are more hydrophilic and larger than the ones of native CDs, allowing a better accommodation capacity.

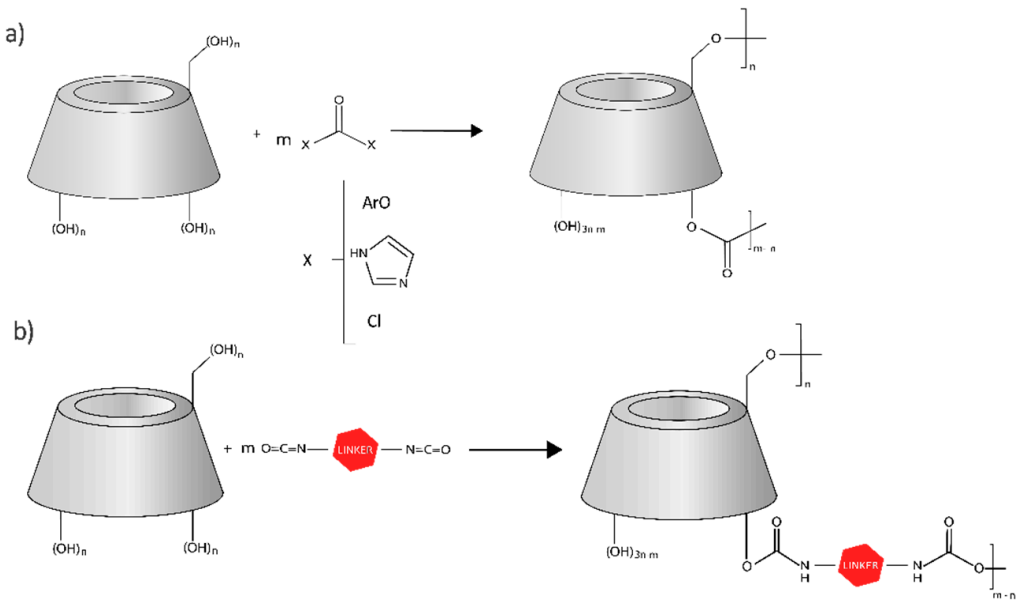

Figure 1. Schematic synthesis of (**a**) CD-based polycarbonate NSs and (**b**) CD-based polyurethane NSs (obtained from [24], published under Creative Commons license).

The typical protocol uses an organic solvent to dissolve the reactants: (i) the CDs, (ii) a catalyst if required and (iii) the suitable amount of the cross-linker. The usual result is a monolithic block or precipitate if the process was sol-gel or precipitation polymerization. In some cases in which the cross-linker is in liquid form and able to solubilize the CD, a

melt polymerization can be performed [29,30]. Finally, the use of water or organic solvents to remove the unreacted substances leads to a dry–solid powder [30–32].

2.2. General Classification

There are four generations of nanosponges that can be described according to their properties and chemical constitution [11]. The first generation of CD-NSs includes one-step reactions between the CD and a cross-linker, whose chemical nature leads to the production of ester, ether, carbonate and urethane nanosponges (Figure 1). The second generation of NSs presents a more complex architecture (pre- or post-functionalization) with different motifs, which results in the obtaining of specific functionalizations, such as charge or luminescence. When adding charge to NSs, the capacity of functionalization is limited by the surface of the polymer and eventual repulsion between charges; however, the addition of luminescence to the NS results in a more uniform distribution. The third generation of nanosponges includes stimulus-sensitive NSs which are able to modulate their behavior according to the environment, for example, regulating the release of a drug. The fourth generation includes molecularly imprinted NSs with a high selectivity towards specific guest molecules.

3. Applications of Cyclodextrin-Based Nanosponges with Proteins and Peptides

The accepted idea of complexation is an adsorption of the peptide into the CD-NS structure, with possible interactions with the hydrophobic residues of the protein by the cavities or hydrophilic residues by the polar groups of the CD-NS (Figure 2). After obtaining the CD-NS correctly, it is time to prepare the protein-loaded CD-NS. The general protocol is classically employed in the case of drug-CD-NSs complexes [24]. The classic mixture of the drug and CD-NS is made in a solvent, commonly water or a buffer solution, for an appropriate time (generally 24 h, but it depends on the adsorption profile) to form the complexes. In this procedure, we must consider the insoluble or soluble nature of our complexes to purify the desired fraction.

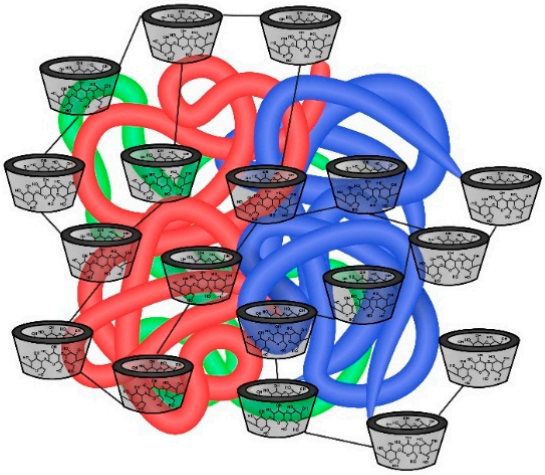

Figure 2. Schematic representation of proteins loaded in CD-NSs. The different chains of the protein are represented in green, red and blue.

Below are some examples of these applications with different proteins and enzymes found in the literature:

3.1. Dioxygenase

In order to test NS capacities, Catechol 1,2-Dioxygenase from *Acinetobacter radioresistens* S13 was used as model [16]. This iron-containing enzyme catalyzes the conversion of catechol into cis, cis-muconate. As support for the enzyme, β-cyclodextrins cross-linked with carbonate groups were used with a ratio of 29 mg of enzyme per gram after incubation

at 22 °C for 4 h using an HEPES pH 8.0 buffer. When studying the activity profile of the enzyme at different levels of the pH and temperature, a shift of the optimal temperature from 30 °C to 50 °C was observed. Likewise, another shift was observed in the optimal pH from 8.5 to 9.5, showing activity even at pH 6.5, where the free enzyme was inactive. The native free form of the enzyme showed a Michaelis–Menten constant, K_M, of 2.0 ± 0.3 μM, whereas for the CD-immobilized enzyme, this value was increased to 16.6 ± 4.8 μM. In contrast, the rate constant, K_{cat}, decreased from 32 ± 2 s^{-1} to 27 ± 3 s^{-1}. The immobilization also produced an increase of the thermostability of the protein, as was shown after several assays. Therefore, after 90 min of incubation at 40 °C, the immobilized enzyme had 60% of its residual activity, while the free enzyme only showed 20%. When the incubation was at 60 °C during 15 min, the free Dioxygenase completely lost its activity, whereas the immobilized enzyme retained 75% of its residual activity. The storage capacity of the enzyme was also increased, as the immobilized Dioxygenase still had 50% of its residual activity after 10 days at 4 °C. The immobilized enzyme also retained activity towards other substrates such as 3- and 4-methylcatechol and 4-chlorocatechol. The retaining of the enzymatic activity allowed the construction of a small Dioxygenase bioreactor, which produced cis, cis-muconic acid from catechol with a high efficiency for 70 days.

3.2. Lipase

Lipase is an enzyme classified as triacylglycerol hydrolase (EC 3.1.1.3) with the biological function to hydrolyze fats through triacylglycerol hydrolysis. However, in the presence of micro-aqueous media, it is able to produce the reverse reaction, trans-esterification [33]. Several studies have demonstrated the capacity of CDs to increase the yield of esterification by the increase of apparent substrate solubility [34,35], even when sometimes used as a support [36,37]. In this last aspect, a non-covalent immobilization on carbonated CD-NSs was tested as a possible support for *Pseudomonas fluorescens* Lipase for the first time [18]. To carry out this adsorption, CD-NSs and *Ps. fluorescens* Lipase were incubated in the presence of a phosphate buffer of pH 8 and Triton X-100. Although urethane NSs presented a higher kinetic adsorption of Lipase (achieving the maximum at 20 h, 12.5 mg Lipase/g), the carbonated CD-NSs presented better activity (6.6-folds higher) and were prolonged in time; in these cases, no enzymatic release was detected after 66 days from the immobilization. The authors suggest this stability and effects might have been caused by (i) the complexation of hydrophobic moieties of the Lipase and (ii) tertiary contacts between hydroxyl CD groups and proteins.

After study of the optimal temperature and pH, the effect of detergents was determined. Usually, immobilized Lipase requires a bit of non-ionic detergent to be activated. The presence of 0.1% Triton X-100 doubled the activity of the immobilized enzyme, with 10 mM of phosphate buffer being the selected ionic strength too.

The activity of the immobilized enzyme in comparison with the free one was measured under continuous-flow conditions plus stirring (slurry conditions). The results demonstrated that the immobilized Lipase retained ~80% activity as compared to the enzyme in solution, achieving the stationary phase in 1 h and the enzyme still being proficient after 3 days in a state of flux, without evidence of a release or yield decrease. The immobilization was able to protect the activity of the enzyme at pH 5; while the free enzyme was instantaneously inhibited, the immobilized enzyme remained at 80% of its activity. As reasonable explanation, the authors claimed a possible additional stabilization of the enzyme due to non-covalent interactions between some aminoacids and nanosponges. Similar results were reported in a study of the effect of methanol, e.g., at 70% *v/v* of methanol, the free enzyme was inactive in comparison with the immobilized, which presented 13% of activity.

3.3. Peroxidase

The enzyme Peroxidase (E.C. 1.11.1.x) has different biological functions, such as to catalyze the oxidation of a variety of organic and inorganic compounds [38]. However, it is commonly used for different purposes, such as immunostaining [39]. In an interesting

study, the team of Fragoso et al. prepared horseradish Peroxidase (HRP) encapsulated in CD-NS immunoconjugates as a signal enhancement tool in optical and electrochemical assays [19]. In this study, pyromellitic nanosponges were used to prepare a particle where the anti-IgG was bonded to CD-NS using N-hydroxysuccinimide and HRP was adsorbed by stirring (62 h, 4 °C, pH 6.5). The results showed that HRP was adsorbed inside the NS, while the antibody remained outside of the particle. Indeed, the dependence of activity on pH showed a considerable influence of the microenvironment, because the optimal range of pH was wider than the free HRP. Even the signal was increased 3.2-fold in the presence of NSs.

3.4. Bovine Serum Albumin

In an interesting article, Swaminathan et al. described the use of poly(amidoamine) nanosponges, named PAA-NS10 and AA-NS11, to complex Bovine Serum Albumin (BSA) as a protein model [17]. These NSs are structurally different compared to the previous carbonated NSs and obtained a 90% (PAA-NS10) and 92% (PAA-NS11) encapsulation efficiency. The values of encapsulation efficiency were increased proportionally in relation with the quantity of PAA-NS added; an increase from 0 to 1% *w/v* of PAA-NS resulted in a maximum increase of 0.3% of the encapsulation efficiency. The slight differences in the encapsulation efficiency seemed to be related to the additional nitrogen atoms or the different crosslinked structure. Finally, the in vitro release showed a much slower release of both NSs against the β-CD/BSA complexes.

3.5. Lysozyme

The same principle was utilized for Lysozyme, an antibiotic enzyme naturally presented in saliva (among others), to improve its stability and bioactivity [20]. Carbonated β-CD nanosponges were employed with three different molar ratios, 1:2, 1:4 and 1:8, but with the addition of $CaCO_3$ and carboxymethyl cellulose to increase the number negative charges (-COO^- groups) to absorb Lysozyme, because its isoelectric point (pI = 11) suggests a higher positive charge at a neutral and slightly alkaline pH. The adsorption and desorption of Lysozyme showed a dependence on temperature, pH, type of NS and formulation, increasing the particle size distribution from 347 to 550 nm. The release profile was even severely affected by the formulation used and affected by the addition of $CaCO_3$, which increased the release percentage. The formulation called 5LF9 (15 mg Lysozyme with 5 mg of β-CDI with $CaCO_3$ and carboxymethyl cellulose) showed the highest release and was able to keep the material stable for 2 months. The bioassay with the bacteria *Micrococcus lysodeikticus* caused the decrease of turbidity as a consequence of the bioactivity.

3.6. Nisin

One of the latest application examples was the study of the natural antimicrobial peptide Nisin by prof. Trotta's group for anticancer and antimicrobial applications [22,23]. In these publications, two different NSs such as BCD-PYRO (1:4) and βCD-CDI (1:4) were used to complex Nisin against two different cell lines: breast and colon cancer cell lines. The loading of Nisin into CD-NSs was proven using the changes in the particle size and Z-potential values; in addition, the complexes showed a slower release of Nisin compared to free-Nisin (7-fold in 5 h). In addition, CD-NSs were able to protect the peptide of specific pepsin degradation, as suggested in the SDS-PAGE study. In both cell lines, the use of BCD-PYRO and βCD-CDI enhanced the cytotoxicity capacity of Nisin, but only with statistical significance between both carriers in colon cancer cells. The study of the specific route of cell toxicity was carried out using flow cytometry, and it occurred by the apoptotic pathway, with late apoptosis/necrosis for colon and early apoptotic for breast cancer.

The antimicrobial enhancement of a Nisin-loaded NS was tested against Gram-positive (*Staphylococcus aureus*) and -negative (*Escherichia coli*) bacteria. The results showed that the antimicrobial activity was significantly increased when the complexed form was used (Figure 3). An interesting point was the total viable counts (TVCs) obtained of cooked

chicken meat sample after treatment, of which the values were considerably lower for 30 days in the presence of complexes; furthermore, nisin in formulations and in contact with pepsin showed better inhibitory behavior on bacterial growth on the cooked chicken meat after 30 days than free-Nisin contacted with pepsin, indicting the protective effect of CD-NSs on the Nisin peptide in presence of pepsin [23].

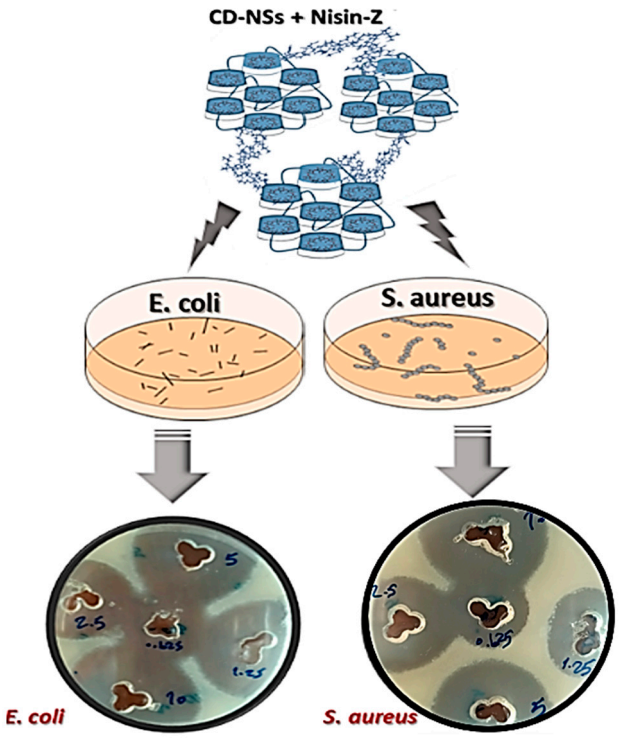

Figure 3. Schematic representation of the anti-bacterial effect of Nisin complexed to CD-NSs.

3.7. Insulin

Insulin is one of the most important proteins used in therapy; for that reason, it was selected as a case study to investigate the use of CD-NS for the oral delivery (Figure 4) of proteins [21]. BCD–PYRO were selected as carriers, increasing the average size by 10% when the complex was formed, but the Z-potential remained the same. Different techniques, such as DSC or FTIR, confirmed the formation of the complex. This NS also demonstrated great muco-adhesion and swelling properties in vitro, properties particularly interesting in drug delivery, because swelling enables the gradual release of drugs, and the muco-adhesion indicates the capacity to be adhered to biological barriers. The complexes showed stability for at least 6 months at 4 °C and great stability during the in vitro digestion, where the release of Insulin was remarkably low at the acidic pH but gradual at pH = 6.8, releasing 10-times more in 2 h. As expected, the presence of NSs increased the internalization of Insulin, as shown by the data of the MTT test, where the presence of Insulin increased the signal due to its activity as a growth factor. The study with fluorescent-labeled NS suggested that the role of NS in oral drug delivery would be its adhesion to the intestinal barrier, due to its mucoadhesive properties, and releasing Insulin slowly due to its swelling state.

Finally, the authors moved this research to in vivo experiments administrating Insulin-loaded NSs via the duodenal route, thus bypassing the activity of the stomach. In this case, BCD–PYRO was able to promote the internalization of Insulin as the cell line experiment demonstrated: the plasma concentration of Insulin was halved after 1 h, followed by a pseudo-plateau for the following 4 h. The fact that no peak of Insulin was found in the rat plasma of the free Insulin group demonstrated that the presence of BCD–PYRO was the principal factor to promote the internalization of Insulin. Finally, no toxicity of this CD-NS was found up to 2000 mg/kg.

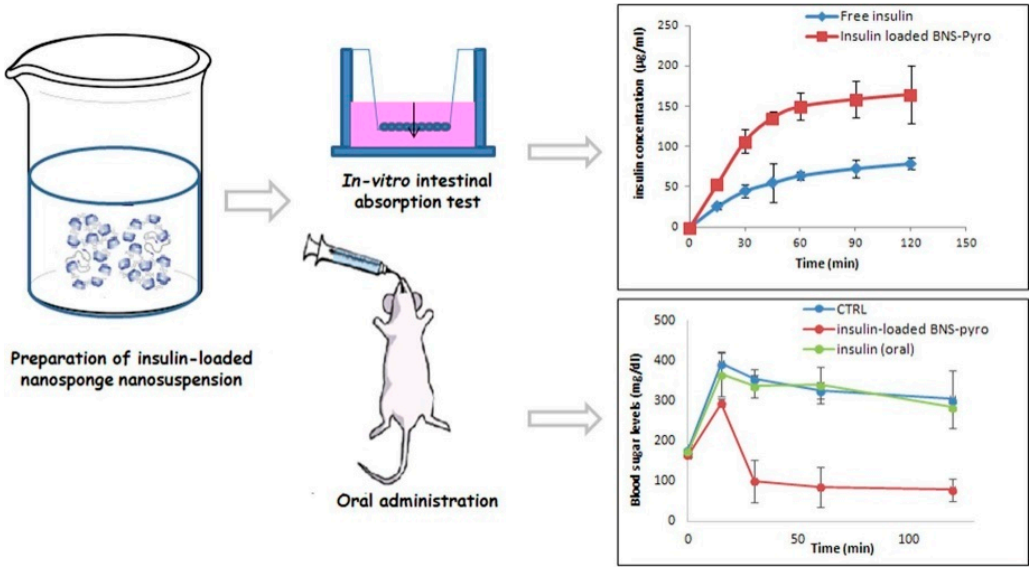

Figure 4. Schematic Insulin case study with the results obtained of absorption (**upper**) and blood glucose concentration (**lower**). Obtained from [21] with permission.

4. Conclusions and Prospects

In this entry, we have shown the promising and real applicability of CD-NSs with proteins. The polymers were able to enhance the specific activity of the enzymes with their reagents to increase their stability and change specific enzymatic values, such as the Km, as consequence of the microenvironment modification, as occurs with enzymatic supports. This behavior suggests interesting applications of CD-NSs as enzymatic supports or immobilizer agents. The studies with BSA and Lysozyme demonstrated the importance of a correct formulation to improve its bioactivity or to obtain a slower release. Moreover, the bioactivity of Lysozyme was increased in comparison with the free sample.

In the case of Nisin, the CD-NS, besides improving its release and stability, as demonstrated by the dialysis studies or the pepsin degradation profile, were shown to increase the anticancer activity of this peptide against two cancer cell lines (breast and colon) and enhance its antibacterial activity against both positive and negative bacteria. Finally, in the Insulin study that was carried out both in vitro and in vivo, the capacity of these polymers as a carrier for proteins was demonstrated. The CD-NSs were also demonstrated to have an interesting mucoadhesive capacity that could improve the bioavailability of drugs or compounds loaded in them, as demonstrated by the negative presence of Insulin in the free Insulin control.

There is no doubt that although the application or type of protein is different, the versatility of CD-NSs to adapt to each protein (thanks to their varied size, charge and capacity) make these materials a promising excipient that will reach new applications in the coming years, maturing the few trials carried out to date in the field of macromolecules such as proteins.

Author Contributions: Conceptualization, S.L.A., Y.K.M., F.J.V.-S., F.C., R.C., F.T. and A.M.; validation, R.C., F.T. and A.M.; formal analysis, S.L.A., Y.K.M. and F.J.V.-S.; investigation, S.L.A., Y.K.M. and F.J.V.-S.; resources, R.C. and F.T.; data curation, S.L.A., Y.K.M., F.J.V.-S., F.C., R.C., F.T. and A.M.; writing—original draft preparation, S.L.A., Y.K.M. and A.M.; writing—review and editing, S.L.A., Y.K.M., F.J.V.-S., F.C., R.C., F.T. and A.M.; visualization, Y.K.M. and A.M.; supervision, F.T. and A.M.; project administration, A.M.; funding acquisition, F.T. and A.M. All authors have read and agreed to the published version of the manuscript.

Funding: Not applicable.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: This work is the result of (1), for Y.K.M., a contract for the University of Turin (Italy) for PhD. Training and (2), for A.M., (i) an aid to postdoctoral training and improvement abroad (for A.M., number 21229/PD/19), financed by the Consejería de Empleo, Universidades, Empresa y Medio Ambiente of the CARM, through the Fundación Séneca-Agencia de Ciencia y Tecnología de la Región de Murcia, and (ii) a RTDA contract from the D.M 1062/2021 (Ministero dell'Università e della Ricerca) for the University of Turin.

Conflicts of Interest: The authors declare no conflict of interest.

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